

The distribution of elastin in developing and adult rat organs using immunocytochemical techniques

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(Accepted 20 December 1988)

INTRODUCTION

Elastin is the major protein of elastic system fibres, the principal connective tissue components responsible for the elasticity of a number of compliant organs such as aorta and skin. The elastic system fibres include elastic fibres, elaunin fibres and oxytalan fibres (Cotta-Pereira, Rodrigo & Bittencourt-Sampio, 1976). The elastic fibre consists of two morphologically distinct components (Ross & Bornstein, 1969): large amounts of an amorphous elastin core surrounded by a few microfibrils which are glycoprotein in nature (Sear, Grant & Jackson, 1981). Elaunin fibres also contain both components but are composed predominantly of microfibrils with little amorphous elastin, whereas oxytalan fibres are, exclusively, bundles of microfibrils (Cotta-Pereira *et al.* 1976). Oxytalan and elaunin fibres appear to be precursors of the mature elastic fibre (Gawlik, 1965).

By light microscopy, demonstration of elastin has relied on characteristic staining reactions, chiefly with orcein, aldehyde-fuchsin and resorcin-fuchsin based stains. These classical stains do not however provide unequivocal identification of elastin as their specificity is now in doubt (Puchtler, Meloan & Pollard, 1976; Fakan & Saskova, 1982), thus limiting the use of these tinctorial dyes. Recently, there have been a number of reports using immunocytochemical detection of elastin by light microscopy (McCullagh, Barnard, Davies & Partridge, 1980; Barnard *et al.* 1982*a*; Barnard, Davies & Young, 1982*b*; Fukuda, Ferrans & Crystal, 1983; Akita, Barrach & Merker, 1986). The sensitivity and specificity of these techniques have allowed more detailed studies of tissues containing relatively small amounts of elastin.

In this study, localisation of elastin in a range of organs from adult and developing rats using immunocytochemical techniques has revealed a wider distribution than has previously been reported.

MATERIALS AND METHODS

Isolation of elastin

The lungs and thoracic aortae from 10 adult rats were subjected to different preliminary treatments. The lungs were homogenised in phosphate buffered saline (PBS) and, after centrifugation, the residue was extracted four times by homogenisation in 2% sodium dodecyl sulphate (SDS). The residue was extracted three times in PBS to remove most of the remaining SDS. This procedure has been shown to remove the majority of the non-crosslinked proteins (Laurent, Cockerill, McNulty & Hastings, 1981). The aortae were rinsed in PBS and ground to a fine powder in a nitrogen mill (Spex Industries Inc., New Jersey, USA). Both the lung and aorta tissues were resuspended three times in acetone, twice in ether and dried. The isolation

procedure was a modification of the method of Rasmussen, Bruenger & Sandberg (1975). The dried material was extracted for 18 hours at 4 °C with 5 M guanidine hydrochloride, pH 7.6 containing 1% (v/v) 2-mercaptoethanol. The residue was further treated with cyanogen bromide (CNBr) in 70% (v/v) formic acid for 4 hours at 30 °C. After the addition of a tenfold excess of water the CNBr was removed by concentrating the suspension to the original volume by rotary evaporation. The elastin was recovered by centrifugation, washed three times with water and freeze dried.

Isolation of soluble elastin

Soluble elastin was isolated from the lungs (wet weight 1.5 g) of young growing rats (60–100 g) by extraction with 0.5 M-NaCl (pH 7.4) containing protease inhibitors essentially as described by Foster *et al.* (1975). The extract was partially purified by salt precipitation. The lung tissue was further extracted with 2% SDS containing 5 mM dithiothreitol, conditions designed to solubilise microfibrillar glycoproteins.

These extracts were analysed by SDS-PAGE using 9% crosslinked gels (Laemmli, 1970) and transferred to nitrocellulose for immunodetection (Towbin, Staehelin & Gordon, 1979).

Amino acid analysis

Samples of elastin were hydrolysed with 6 M-HCl for 48 hours and aliquots corresponding to 40 µg of protein were analysed using a Hilger Analytical Chromaspeck Amino Acid Analyser.

Preparation of antibodies

Although it was originally planned to raise antibodies to insoluble lung and aorta elastin, only the material isolated from aorta was amenable to suspension in PBS and formation of a stable emulsion with adjuvant. The flocculent nature of the lung elastin effectively prevented this procedure. Consequently, lung elastin was partially solubilised by refluxing with 0.25 M oxalic acid for 45 minutes (Partridge, Davis & Adair, 1955). The suspension was dialysed against water and freeze dried; this material was then used for immunisation. In some experiments aorta elastin was also partially solubilised with 0.25 M oxalic acid and this material, after dialysis and freeze-drying, was used for immunisation (see Discussion).

Rabbits were injected at multiple sites subcutaneously with 0.5–1.0 mg of either lung or aorta elastin suspended in an equal volume of PBS and Freund's complete adjuvant. At 1 month intervals the rabbits were injected twice more with a similar amount of elastin in Freund's incomplete adjuvant. The rabbits were bled 2 weeks after the final injection. For testing the antisera, complete solubilisation of elastin from lung and aorta was achieved by four successive treatments with oxalic acid as described above. These α -elastin preparations were used both for coating 96-well microtitre plates (1 µg/well) and for the preparation of immunoaffinity columns by covalent linkage to Sepharose 4B (1 mg per ml of gel). The antisera were tested by ELISA and, for those with a useful titre, the antibodies were purified by affinity chromatography.

The specificity of the affinity purified antibodies was assessed by ELISA using microtitre plates coated with native and denatured forms of Types I, III, IV and VI collagen, fibronectin, laminin and nidogen.

Immunostaining

Skin, heart, skeletal muscle, kidney, liver, trachea, xiphoid process and hyaline cartilage from the tibia and the head of the femur were dissected from adult male

Hooded Lister rats. Pieces of fresh tissue were immediately frozen in isopentane precooled with liquid nitrogen. Whole body sections of the thorax which included the skin were obtained from 12 to 20 days old fetal rats and were frozen in a similar way. Cryostat sections 5 μm in thickness were cut, air dried and were fixed in acetone for 5 minutes. The sections were pretreated with normal goat serum (1:20 dilution) followed by an overnight incubation at 4 °C with affinity purified antibodies against elastin diluted to 13 $\mu\text{g}/\text{ml}$. After thorough washing the sections were incubated with TRITC labelled goat antirabbit IgG (Sigma, Dorset, UK) for 1 hour. The sections were then washed and mounted in Citifluor (Agar Scientific, Essex, UK) and examined with a Leitz fluorescence microscope. All washings and antibody dilutions were with PBS, pH 7.4 and all of the antibody solutions were centrifuged before use. Some sections of trachea were preincubated before staining with 2% (w/v) testicular hyaluronidase (Type IV-S; Sigma, Dorset, UK) in PBS for 30 minutes at room temperature in order to cleave, if present, any masking glycosaminoglycans (Cotta-Pereira, Del-Caro & Montes, 1984). Control sections were incubated with appropriately diluted normal rabbit serum and some sections were also pretreated with 0.01% (w/v) elastase (Type 1; Sigma, Dorset, UK) in 0.2 M Tris-HCl buffer, pH 8.8 for 1 hour at 37 °C. Immunostained preparations were photographed using Ilford XP1 400 film.

RESULTS

Antibody specificity

Sera from rabbits injected with insoluble aortic elastin reacted very weakly in ELISA plates coated with soluble aorta or lung elastin. Antisera from rabbits injected with partially solubilised lung elastin, however, reacted strongly in ELISA plates coated with both types of soluble antigens. Only these antisera were affinity purified and used for immunocytochemistry. The affinity purified antibodies as expected reacted strongly in ELISA plates coated with elastin but there was no cross reaction with native and denatured collagens, fibronectin, laminin or nidogen. The antibody reacted with soluble elastin; Western blotting of an electrophoretic separation of partially purified tropoelastin from rat lung revealed a single band at about M_r 70000 (not shown). Furthermore similar analyses of lung tissue solubilised under reducing and denaturing conditions showed only the tropoelastin band and no higher- M_r components.

Amino acid analysis

The amino acid profile of lung elastin was typical for elastin in that it contained no methionine, cysteine or hydroxylysine and had proportionately high amounts of valine and alanine. Assessment by the semi-quantitative index of Soskel & Sandberg (1983) indicated a score of 5 out of a possible 8.

Immunofluorescence staining

Heart and skeletal tissue

Elastin was prevalent around the endocardial surface of both ventricles (Fig. 1a) and atria with no apparent difference in the staining pattern of the four chambers. The epicardium, which forms the outer surface of the heart, was also found to stain positively (Fig. 1b). In the myocardium the coronary arteries that are situated in the perimysium displayed abundant elastin staining. Both the inner elastic membrane and adventitia stained strongly, with the media showing a more patchy appearance (Fig. 1c); the perimysium itself was unstained. In the endomysium there was a virtually complete elastin network surrounding the individual myocytes but the capillaries,

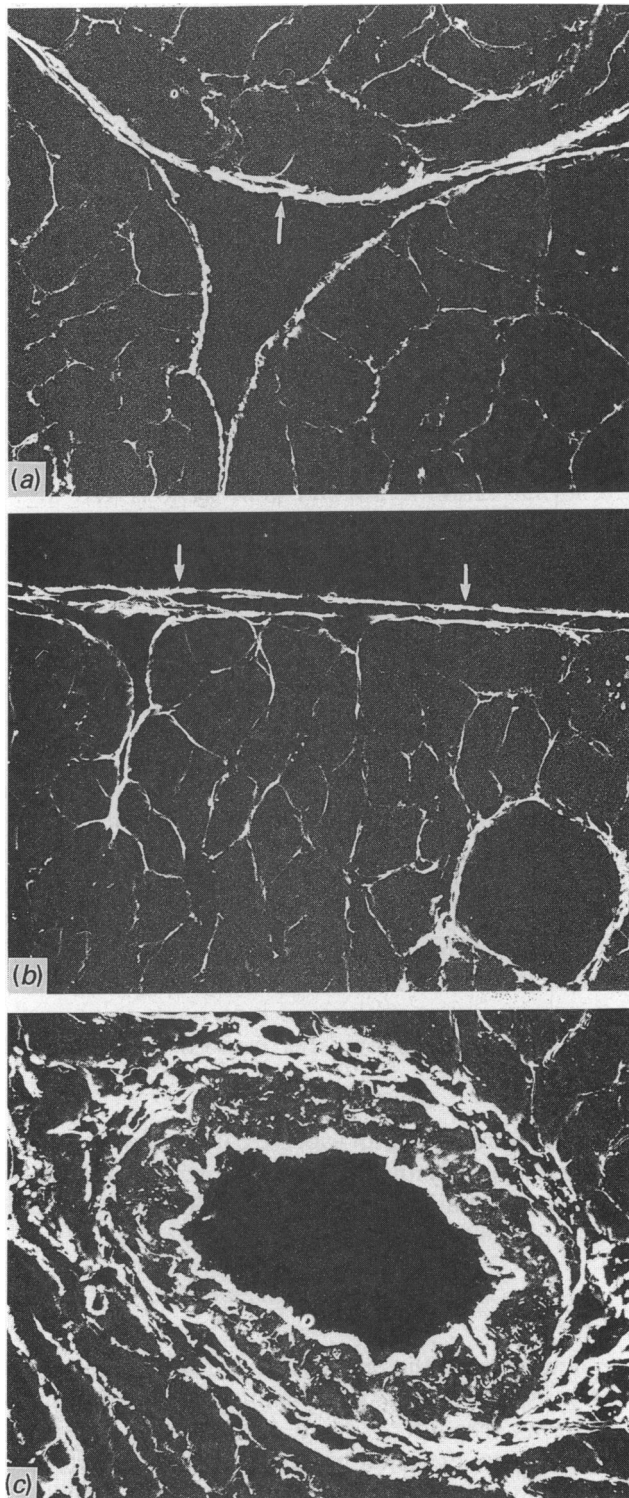


Fig. 1(a-c). Immunostaining of heart tissue. (a) Endocardial surface of left ventricle (arrow). (b) Epicardial surface of left ventricle (arrows). (c) Coronary artery showing strong staining of inner elastic membrane, media and adventitia. $\times 275$.

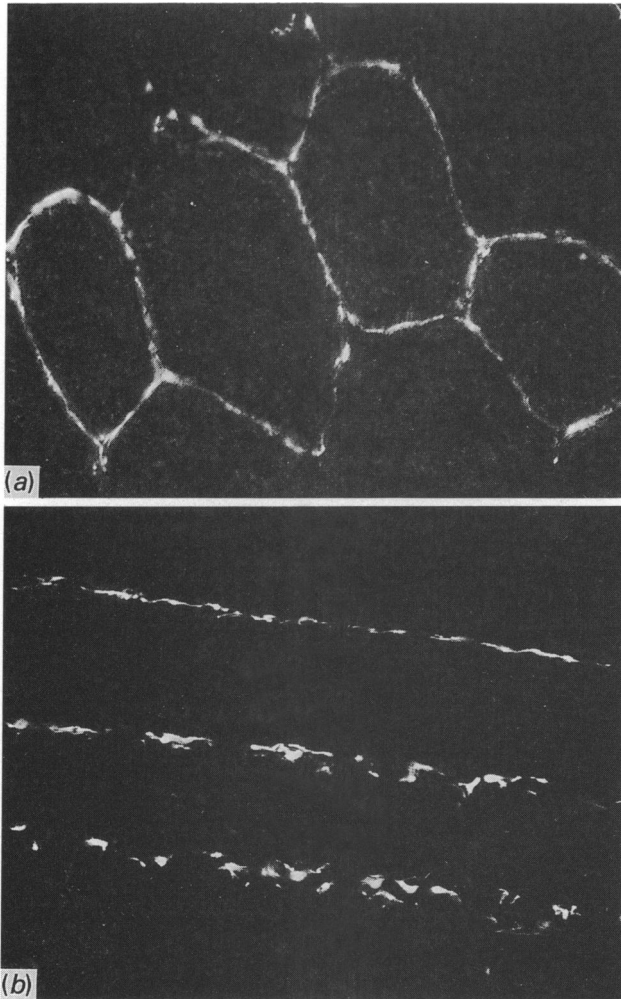


Fig. 2(a-b). Immunostaining of skeletal muscle. Endomysium seen in (a) transverse and (b) longitudinal sections. $\times 430$.

visualised by staining with antibodies to Type IV collagen (not shown), situated in this region were unstained (Fig. 1 a, b). In skeletal muscle, elastin had a similar distribution with the endomysium of the individual fibres strongly stained (Fig. 2a, b).

Cartilage

Tracheal hyaline cartilage was completely unstained, although there was positive staining of the perichondrium together with both the outer fibroelastic layer and the mucosal membrane on the luminal side (Fig. 3a). Pretreatment of the sections with 2% hyaluronidase did not result in any staining of the cartilage. The articular cartilage of the tibia and femur were also unstained (Fig. 3b). The slight staining of the chondrocytes was due to non-specific staining by the TRITC-labelled second antibody, as this staining pattern was also present in the controls. In contrast to these negative results, the hyaline cartilage of the xiphoid process stained strongly. There was intense elastin staining in the matrix which appeared to encircle the individual chondrocytes, and the perichondrium on both sides of the cartilage also stained positively (Fig. 3c).

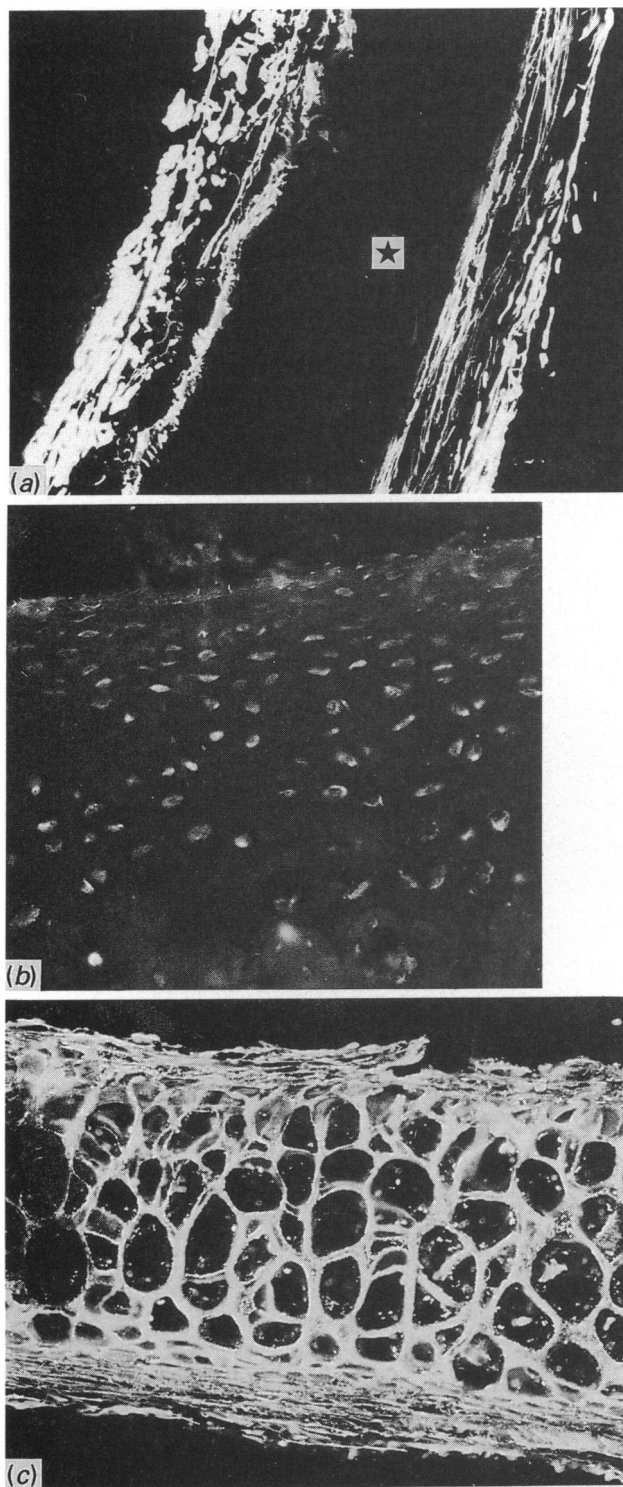


Fig. 3(a-c). Immunostaining of cartilage. (a) Tracheal hyaline cartilage (★) completely unstained. (b) Articular cartilage unstained with non-specific staining of the chondrocytes. (c) Hyaline cartilage of xiphoid process stained strongly. $\times 275$.

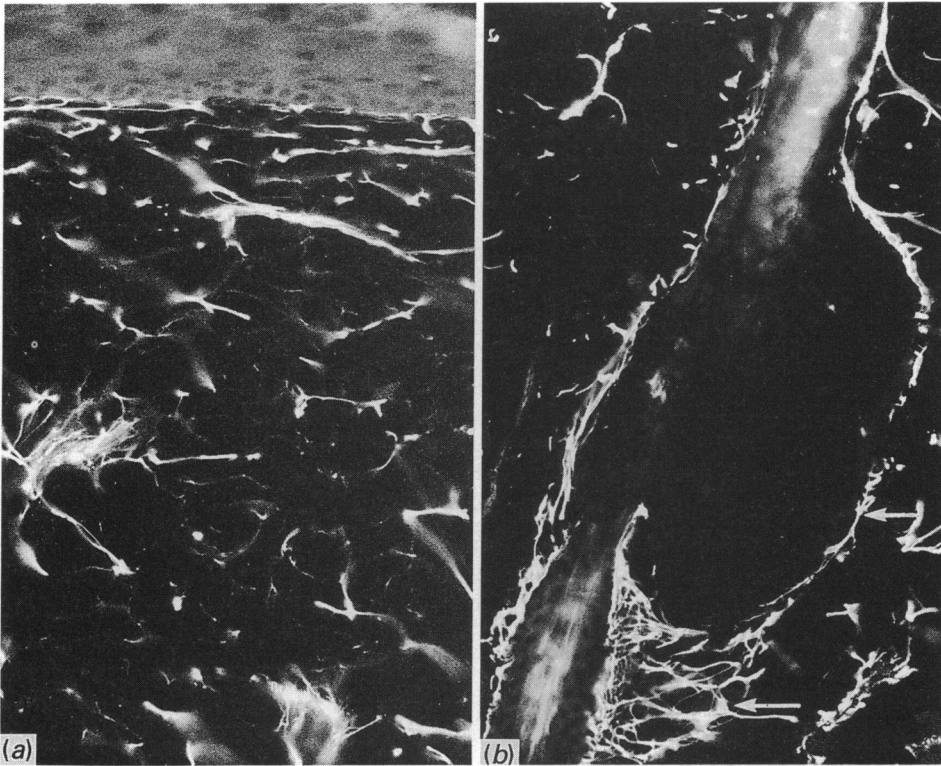


Fig. 4(a-b). Immunostaining of adult skin. (a) Elastin is randomly orientated in the superficial dermis and is closely associated with the epidermis. (b) Strong staining associated with the connective tissue sheath of the hair follicles and sebaceous glands (arrows). $\times 275$.

Skin

Elastin was found predominantly in the superficial and middle dermis with the deeper dermis virtually free from any staining. In the superficial dermis the randomly orientated elastin fibres abutted the underside of the epidermis (Fig. 4a). Elastin was also detected in the connective tissue sheath of the hair follicle and the adjoining sebaceous glands, but was particularly localised around the hair root (Fig. 4b). The hair shaft tended to display some non-specific staining. Although the hypodermis was virtually free of staining the large blood vessels located in this region stained strongly (not shown).

Development of elastin in skin

In fetal rat skin no staining was present in 12 or 14 day gestation rats (Fig. 5a) and sparse fibrillar staining associated with the dermal region was first detected in 16 days old animals (Fig. 5b). Non-specific fluorescence of the epidermis (Fig. 5c), evident at older stages of development and in adult rats, was not present at this stage. At 17 days, staining in the dermal layer associated with the epidermis was more pronounced and a definite pattern was evident. Under the superficial dermal staining there was an area with diffuse fluorescence which, in turn, was underlined by a band of fibrillar staining (Fig. 5d). At 18 days (Fig. 5e), hair follicles were more pronounced and the staining pattern was similar to 19 and 20 days old fetal rats (Fig. 5f) and to that seen in the adult rats. From 18 days onwards, the elastin was confined to the superficial and

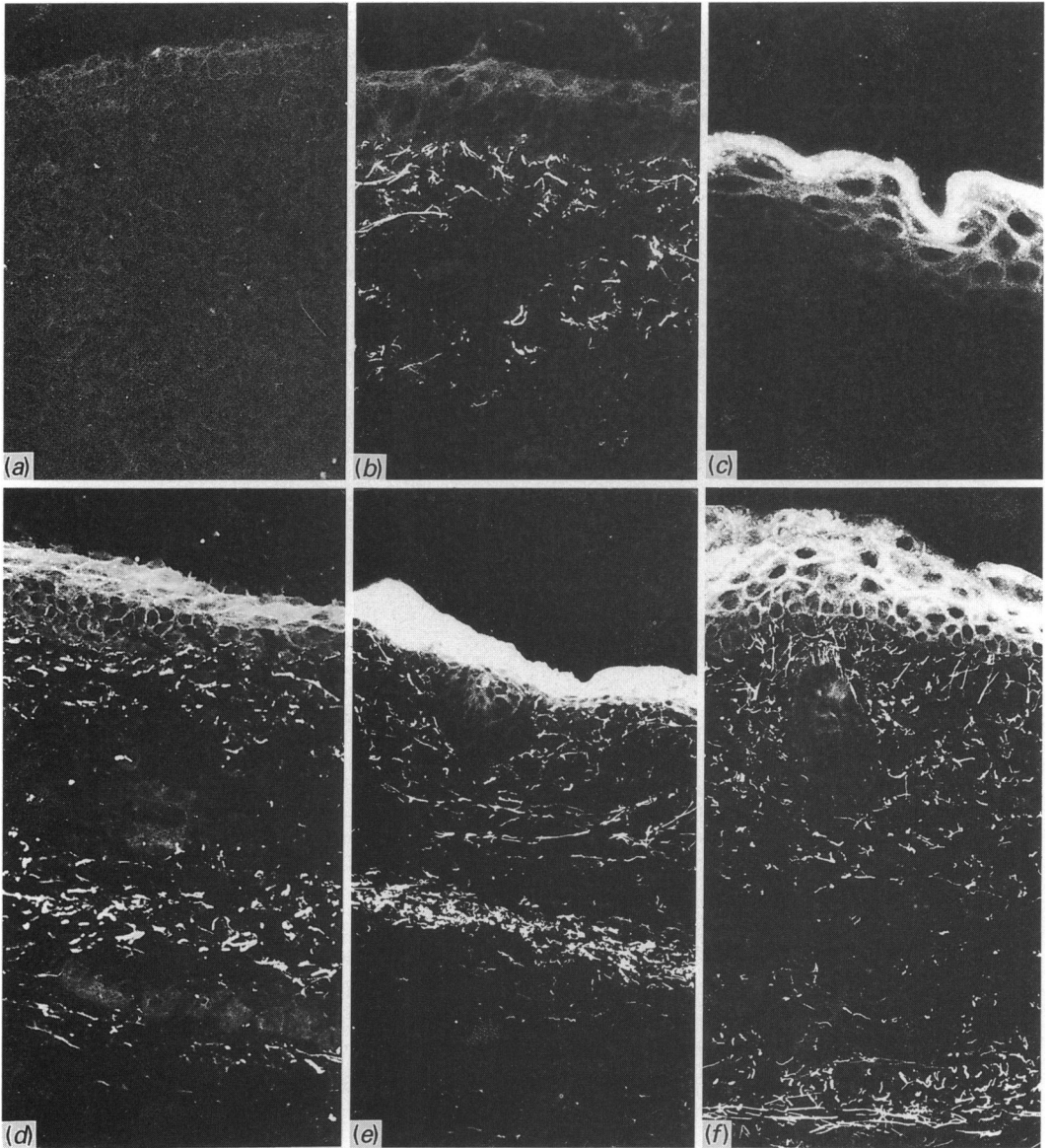


Fig. 5(a-f). Immunostaining of developing rat skin. (a) 14 days; (b) 16 days; (c) 18 days control section; (d) 17 days; (e) 18 days; (f) 20 days. (a, d, e, f) $\times 275$; (b, c) $\times 430$.

middle dermis as in the adult rats. The loose band of staining seen at 17 days appeared to have aggregated to form a distinct line of fluorescence running under both the dermis and the hypodermis.

Liver and kidney

In the liver there was no staining of the hepatic parenchyma with staining limited to the blood vessels of the portal area. A similar distribution was seen in the kidney with positive staining of the blood vessels but the glomeruli, distal and proximal tubules were unstained (Fig. 6).

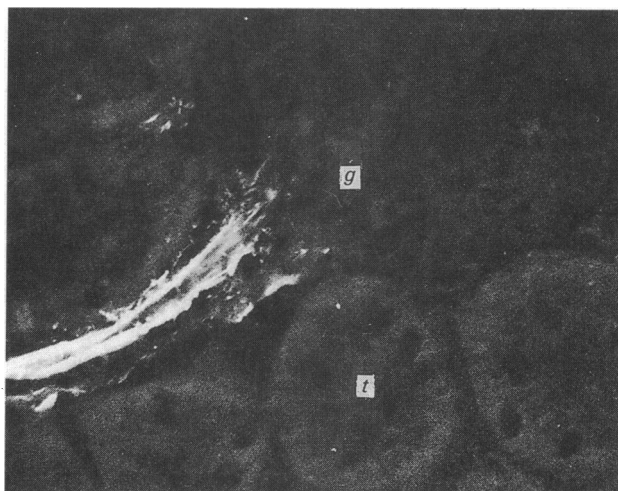


Fig. 6. Immunostaining of kidney. Arteriole stained, glomerulus (g) and tubules (t) unstained. $\times 275$.

Control sections

Sections incubated with normal rabbit serum or treated with elastase prior to staining displayed no positive staining (not shown).

DISCUSSION

For many years elastin was regarded as non-antigenic (Ayer & Feldamis, 1958; Walford, Hirose & Doyle, 1959). However, it was shown that, using soluble elastin as the immunogen, antibodies of low titre could be raised in rabbits (Jackson, Sandberg & Cleary, 1966). More recently, antisera have been successfully raised against insoluble elastin (Sykes & Chidlow, 1974; Houle & LaBella, 1977), soluble elastin (Houle & LaBella, 1977; Barnard *et al.* 1982a) and to the elastin precursor, tropoelastin (Daynes, Thomas, Alvarez & Sandberg, 1977; Daga-Gordini, Bressan, Castellani & Volpin, 1987). Varying success has been reported in raising antisera to insoluble elastin in rabbits (Darnule, Likhite, Turino & Mandl, 1977; Mecham & Lange, 1980; Kucich, Christner, Rosenbloom & Weinbaum, 1981) and an improved response is often achieved in sheep (Sykes & Chidlow 1974; Kucich *et al.* 1981). In the present study, very low titres were obtained after immunisation with insoluble aortic elastin and even after boosting these animals with partially solubilised material (as for lung elastin), only moderately increased titres were obtained. The reasons for this apparent tissue difference in the immunogenicity of elastin are unclear.

The immunofluorescence results indicated that elastin is abundant in the extracellular matrix (ECM) of both cardiac and skeletal muscle. Although the ECM of these two tissues has been studied extensively, most of the data have focused on the collagen content and its distribution within these tissues. Previous studies indicated that the elastic fibres within the myocardium formed a network around the individual myocytes (Nagal, 1945; Puff & Langer, 1965; Robinson, Cohen-Gould & Factor, 1983). Our results substantiate these findings but demonstrate more clearly that elastin is a major component of the cardiac endomysium. By chemical analysis, Bendall (1967) estimated that, in bovine muscle, elastin constituted approximately 5–40% of the total connective tissue and non-specific staining techniques indicated that it was

located in the peri- and epimysium. The results of specific immunostaining in the present study demonstrate that elastin is a significant component of the endomysium, and it is probable that elastin together with collagen plays a major role in the structural and mechanical properties of both cardiac and skeletal muscle.

Our results also indicated that elastin is present on the endocardial surface of both ventricles and atria of the heart. A similar distribution was demonstrated in the left atria using classical elastica staining techniques and electron microscopy (Klein & Böck, 1983). Contrary to the present results, however, these workers reported very little, if any, elastica-positive material associated with the ventricle endocardium and also found only microfibrillar material associated with the right atrium.

There are conflicting reports on the presence of elastic system fibres in the specific types of cartilage, particularly hyaline cartilage. It has been demonstrated that elastic fibres are present within elastic cartilage (Anderson, 1964; Keith, Paz, Gallop & Glimcher, 1977) and that elastin is synthesised by the chondroblasts (Quintarelli *et al.* 1979). Our results for hyaline cartilage indicate that, in agreement with others (Anderson, 1964; Cotta-Pereira *et al.* 1984), there is no elastin associated with cartilage in the trachea. The antibodies used in the present study appear to be completely unreactive towards microfibrillar glycoproteins, consistent with the lack of staining of oxytalan fibres that have been localised histochemically in hyaline cartilage (Cotta-Pereira *et al.* 1984). The lack of detection of the elastin in elaunin fibres that have been reported in tracheal cartilage (Gawlik, 1965; Cotta-Pereira *et al.* 1984) suggests that, in the present study, unmasking of the protein was not achieved even after hyaluronidase treatment. A recent ultrastructural study, however, has shown that hyaline articular cartilage contains small elastic fibres which are distinct from elaunin fibres (Hesse, 1987). It is possible due to their small size that these structures are, in fact, elaunin fibres and not detected in this study for reasons previously discussed. The presence of elastin in the hyaline cartilage of the xiphoid process suggests that this tissue should be considered as an elastic cartilage.

This study demonstrated that in adult rat skin the distribution of elastin differs from that in man where elastin is found in the middle and deep dermis with oxytalan fibres forming a network perpendicular to the dermo-epidermal junction (Frances & Robert, 1984). We have demonstrated that in the rat, elastin predominates in the superficial and middle dermis with the deeper dermis bordering the hypodermis essentially free of elastin.

Our results on the developing rat skin indicate that elastin is first synthesised in the skin at Day 16 of embryonic life. Elastin biosynthesis after this period appeared to be very rapid with its distribution taking the adult form on Day 18. The appearance of elastin at Day 16 does not preclude the presence of microfibrils which are known to be produced before the amorphous elastin is secreted (Ross & Bornstein, 1969). Fetal elastin may contain antigenic sites that are not present in the mature protein (Barnard *et al.* 1982a) and consequently are not recognised by the antibody. It has been postulated that elastin has a developmental as well as a mechanical role within tissues (Mecham, Griffin, Madaras & Senior, 1984). These workers demonstrated that fibroblasts displayed a chemotactic response to elastin which coincided with the cells' ability to synthesise the protein. The present results suggest that any developmental migration of fibroblasts elicited by elastin synthesis may occur at 16 days of embryonic life in the rat skin.

SUMMARY

The localisation of elastin in a number of rat organs was carried out by immunocytochemical techniques, using antibodies raised in rabbits to partially solubilised lung elastin. The results demonstrated that in cardiac and skeletal muscle, elastin was found in the endomysium surrounding the individual myocytes and fibres respectively, a localisation that suggests elastin may have important structural and mechanical roles in muscle. Elastin was located in the hyaline cartilage of the xiphoid process but not in hyaline articular or tracheal cartilage. In the developing skin, elastin was first detected in 16 days old embryos which took on the adult appearance at Day 18. In the adult skin, elastin was found predominantly in the superficial and middle dermis with accumulation round the skin appendages. Elastin was detected in the kidney and liver only in association with the blood vessels.

The authors are grateful to Mr David Brown for the amino acid analysis of elastin and Mr George Milne for assistance with the electrophoresis and immunoblotting procedures.

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